PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C12N 15/31, C07K 13/00
C12N 1/21, A61K 39/10

A1

(11) International Publication Number: WO 92/17587

(43) International Publication Date: 15 October 1992 (15.10.92)

(21) International Application Number: PCT/GB92/00561

(22) International Filing Date: 27 March 1992 (27.03.92)

(30) Priority data:
9106568.0 27 March 1991 (27.03.91) GB

(71) Applicant *(for all designated States except US)*: THE WELLCOME FOUNDATION LIMITED [GB/GB]; Unicorn House, 160 Euston Road, London NW1 2BP (GB).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)* : CHARLES, Ian, George [GB/GB]; The Wellcome Foundation Limited, Langley Court, Deckenham, Kent BR3 3BS (GB).

(74) Agents: WOODS, Geoffrey, Corlett et al.; J.A. Kemp & Co., 14 South Square, Gray's Inn, London WC1R 5LX (GB).

(81) Designated States: AT (European patent), BE (European patent), CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), MC (European patent), NL (European patent), SE (European patent), US.

Published
With international search report.

(54) Title: BORDETELLA BRONCHISEPTICA OUTER MEMBRANE ANTIGEN

DWNNQSIIKAGERQHGIHIKQSDGAGVRTATGTTIKVSGRQAQGVLLENPAAELRFQNG
SVTSSGQLFDEGVRRFLGTVTVKAGKLVADHATLANVSDTRDDDGIALYVAGEQAQASI
ADSTLQGAGGVRVERGANVTVQRSTIVDGGLHIGTLQPLQPEDLPPSRVVLGDTSVTAV
PASGAPAAVSVFGANELTVDGGHITGGRAAGVAAMDGAIVHLQRATIRRGDAPAGGAVP
GGAVPGGFGPLLDGWYGVDVSDSTVDLAQSIVEAPQLGAAIRAGRGARVTVSGGSLSAP
HGNVIETGGGARRFPPPASPLSITLQAGARAQGRALLYRVLPEPVKLTLAGGAQGQGDI
VATELPPIPGASSGPLDVALASQARWTGATRAVDSLSIDNATWVMTDNSNVGALRLASD
GSVDFQQPAEAGRFKCLMVDTLAGSGLFRMNVFADLGLSDKLVVMRDASGQHRLLVRNS
GSEPASGNTMLLVQTPRGSAATFTLANKDGKVDIGTYRYRLAANGNGQWSLVGAKAPPA
PKPAPQPGPQPGPQPPQPPQPPQPPQRQPEAPAPQPPAGRELSAAANAAVNTGGVGLAS
TLWYAESN (I)

(57) Abstract

A protein which is uncontaminated by components from *B. bronchiseptica*, which is capable of binding to antibody which also binds the native P.68 antigen of *B. bronchiseptica* and which has (a) the amino acid sequence (I) or (b) an amino acid sequence which has a homology of more than 98 % with the said amino acid sequence (a).

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MG	Madagascar
AU	Australia	FI	Finland	ML	Mali
BB	Barbados	FR	France	MN	Mongolia
BE	Belgium	GA	Gabon	MR	Mauritania
BF	Burkina Faso	GB	United Kingdom	MW	Malawi
BG	Bulgaria	GN	Guinea	NL	Netherlands
BJ	Benin	GR	Greece	NO	Norway
BR	Brazil	HU	Hungary	PL	Poland
CA	Canada	IT	Italy	RO	Romania
CF	Central African Republic	JP	Japan	RU	Russian Federation
CG	Congo	KP	Democratic People's Republic of Korea	SD	Sudan
CH	Switzerland			SE	Sweden
CI	Côte d'Ivoire	KR	Republic of Korea	SN	Senegal
CM	Cameroon	LI	Liechtenstein	SU	Soviet Union
CS	Czechoslovakia	LK	Sri Lanka	TD	Chad
DE	Germany	LU	Luxembourg	TG	Togo
DK	Denmark	MC	Monaco	US	United States of America

BORDETELLA BRONCHISEPTICA OUTER MEMBRANE ANTIGEN

This invention relates to proteins suitable for use in vaccination against Bordetella bronchiseptica.

Bordetalla bronchiseptica is a bacterial pathogen associated with respiratory diseases in animals, particularly atrophic rhinitis in pigs. The disease state is recognised by severe changes in the nasal architecture of developing piglets, pneumonia and growth retardation. We have demonstrated that protection against B.bronchiseptica mediated atrophic rhinitis correlates with the presence of an outer membrane protein with a molecular weight of 68kDa (P.68) as determined by SDS polyacrylamide gel electrophoresis (Novotny et al., 1985b). Antibodies to this 68kDa protein can be detected in high titre in protected piglets, while the titre is low or absent from non-protected animals (Novotny et al., 1985b). Furthermore, a naturally occurring mutant B.bronchiseptica lacking the 68kDa protein is unable to protect piglets when used as a vaccine and is unable to induce pathological changes in infected piglets (Novotny et al., 1985b).

Administration of a vaccine consisting of a purified preparation of the 68kDa protein to pregnant sows results in significant protection of the resulting piglets when challenged with virulent B.bronchiseptica (Kobisch & Novotny, 1990). In addition it has been demonstrated that passively administered monoclonal antibody BB05 (Novotny et al., 1985a) specific for the 68kDa protein is able to prevent death from pneumonia and development of atrophic rhinitis in mice that had been aerosol infected with a virulent strain of B.bronchiseptica.

Native P.68 antigen can only be purified at low levels from cultures of B.bronchiseptica. We have now found that the gene from which P.68 is expressed in fact encodes a protein with a Mr of 93996 (P.94). It may be surmised that P.94 is processed to form P.68 on the surface of B.bronchiseptica. We have also found that heterologous

expression of the full-length gene encoding P.94 in E.coli results in similar processing, with the P.68 antigen targeted to the bacterial outer membrane. This allows P.68 to be produced in large amounts uncontaminated by other B.bronchiseptica components.

Accordingly, the present invention provides a protein which is uncontaminated by components from B. bronchiseptica, which is capable of binding to antibody which also binds the native P.68 antigen of B. bronchiseptica and which has (a) the amino acid sequence:

DWNNQSIIKAGERQHGIHIKQSDGAGVRTATGTTIKVSGRQAQGVLLENPAAELRFQNG
SVTSSGQLFDEGVRRFLGTVTVKAGKLVADHATLANVSDTRDDDGIALYVAGEQAQASI
ADSTLQGAGGVRVERGANVTVQRSTIVDGGLHIGTLQPLQPEDLPPSRVVLGDTSVTAV
PASGAPAAVSVFGANELTVDGGHITGGRAAGVAAMDGAIVHLQRATIRRGDAPAGGAVP
GGAVPGGFGPLLDGWYGVDVSDSTVDLAQSIVEAPQLGAAIRAGRGARVTVSGGSLSAP
HGNVIETGGGARRFPPPASPLSITLQAGARAQGRALLYRVLPEPVKLTLAGGAQGQGDI
VATELPPIPGASSGPLDVALASQARWTGATRAVDSLSIDNATWVMTDNSNVGALRLASD
GSVDFQQPAEAGRFKCLMVDTLAGSGLFRMNVFADLGLSDKLVVMRDASGQHRLLVRNS
GSEPASGNTMLLVQTPRGSAATFTLANKDGKVDIGTYRYRLAANGNGQWSLVGAKAPPA
PKPAPQPGPQPGPQPPQPPQPPQPPQRQPEAPAPQPPAGRELSAAANAAVNTGGVGLAS
TLWYAESN

or (b) an amino acid sequence which has a homology of more than 98% with the said amino acid sequence (a).

The protein may be isolated and purified. It may therefore be provided in pure form. Purity may be 90% or more, or 95% or more. Amino acid sequence (a) is the amino acid sequence P.68. Amino acid sequence (b) may contain one or more amino acid insertions, deletions or substitutions. Amino acid sequences (a) and (b) may therefore differ from each other at no more than twelve positions, for example at no more than eight positions or at no more than four positions. The homology between the sequences may therefore by 98% or more, for example 99% or more.

One or more of the amino acid residues of amino acid sequence (a) may therefore be deleted or substituted or one

or more additional amino acid residues may be inserted, provided the physicochemical character of the original sequence is retained for example in terms of charge density, hydrophobicity/hydrophilicity, size and configuration. A protein composed of the modified amino acid sequence must also be capable of binding to antibody which is capable of binding the native P.68 antigen. Candidate substitutions are:

A for G and vice versa;

V by A, L or G;

K by R;

S by T and vice vera;

E by D and vice versa and

Q by N and vice versa.

A protein of the invention is obtained by recombinant DNA technology. The preparation of the protein therefore depends upon the provision of a DNA sequence encoding protein. However, we have found that the protein of the invention is a processed form of a larger precursor protein designated P.94. A DNA sequence encoding P.94 may also be used to express a protein of the invention. In further aspects, therefore, the invention provides:

- a DNA sequence which encodes a protein of the invention composed of amino acid sequence (a) or (b);
- a DNA sequence which consists essentially of the nucleotide sequence shown in Figure 1 from nucleotide 247 to nucleotide 2040;
- a DNA sequence which differs from the nucleotide sequence shown in Figure 1 from nucleotide 247 to nucleotide 2040 at no more than twelve positions;
- a protein which as the amino acid sequence shown in Figure 1 or an amino acid sequence which has a homology of more than 98% with the amino acid sequence shown in Figure 1;
- a DNA sequence which encodes a protein as defined immediately above;

- a DNA sequence which consists essentially of the nucleotide sequence shown in Figure 1 from nucleotide 145 to nucleotide 2877;
- a DNA sequence which differs from the nucleotide sequence shown in Figure 1 from nucleotide 145 to 2877 at no more than twelve positions;
- a DNA sequence which consists essentially of the sequence shown in Figure 1 or a sequence which has a homology of more than 98% with the sequence shown in Figure 1.

The nucleotide sequences shown in Figure 1 from nucleotide 247 to 2040 and from nucleotide 145 to 2877 encode P.68 and P.94 respectively. DNA sequences which contain differences from these two sequences may have differences at up to twelve nucleotide positions, for example at no more than eight positions or at no more than four positions. These differences may or may not result in coding changes. Codons may be inserted or omitted, and nucleotides may be substituted. There may be a degree of homology of 98% or more, for example 99% or more, between the sequences shown in Figure 1 which encode P.68 and P.94 and modified versions of these DNA sequences.

A DNA sequence may be purified and isolated. A DNA sequence may be synthesised de novo, for example by synthesising and annealing oligonucleotides of appropriate sequences. A DNA sequence may be a cloned sequence.

The cloning of the DNA sequence may be carried out using standard procedures known in the art. However, it is particularly advantageous in such procedures to employ the sequence data disclosed herein so as to facilitate the identification and isolation of the desired cloned DNA sequences. Preferably, the DNA is isolated by the method described in Hull et al. (1981) as modified by Maskell et al., J. Bacteriol. 170: 2467-2471 (1988). The DNA is then digested with a restriction enzyme to generate short fragments which are then inserted into a cloning vector,

such as the cosmid pHC79 or a derivative thereof and the resulting recombinant DNA molecules used to transform E.coli and thus generate the desired library.

The library may be screened using a standard screening strategy. For example one may employ as hybridisation probes one or more labelled oligonucleotides synthesised using the DNA sequence information disclosed herein. One or more additional rounds of screening of one kind or another may be carried out to characterise and identify positive clones.

Having identified a first positive clone, the library may be rescreened for additional positive clones using the first clone as a hybridisation probe. Alternatively or additionally, further libraries may be prepared and these may be screened using hybridisation probes. In this way, further DNA sequences may be obtained.

Thus cloned or synthesised, the desired DNA sequences may be inserted into an expression vector using known and standard techniques. The expression vector is normally cut using restriction enzymes and the DNA sequence inserted using blunt-end or staggered-end ligation. The cut is usually made at a restriction site in a convenient position in the expression vector such that, once inserted, the DNA sequence is under the control of the elements of DNA that effects its expression.

These elements may vary according to the host but usually include a promoter, ribosome binding site, translation start and stop sites, and a transcriptional termination site. Examples of such vectors include plasmids and cosmids. Expression vectors of the present invention encompass both extrachromosomal vectors and vectors that are integrated into the host cell's chromosome.

The invention therefore also provides

- an expression vector which contains a DNA sequence as herein defined, and which, when provided in a

suitable host, is capable of expressing encoded protein of the invention; and

- a host transformed with such an expression vector.

Examples of host cells of use with the invention include prokaryotic and eukaryotic cells, such as bacterial, yeast, mammalian and insect cells. Particular examples of such cells are E.coli, S.cerevisiae, P. pastoris, Chinese hamster ovary and mouse cells, and Spodoptera frugiperda and Tricoplusia ni. The choice of host cell may depend on a number of factors but, is post-translational modification of the antigen is important, then a prokaryotic host would be preferred.

Transformation of a host cell may be carried out using standard techniques. A phenotypic marker is usually employed to distinguish between the transformants that have successfully taken up the expression vector and those that have not. Culturing of the transformed host cell and isolation of the antigen may also be carried out using standard techniques.

The invention therefore provides a process for the preparation of a protein of the invention, which process comprises maintaining a host transformed with an expression vector according to the invention under such conditions that the said protein is expressed. In more detail, the process may comprise:

- cloning or synthesising a DNA sequence encoding the protein as herein defined;
- inserting the DNA sequence into an expression vector such that it is capable in an appropriate host of being expressed;
- transforming a host cell with the expression vector;
- culturing the transformed host cell; and
- isolating the protein.

In this way heterologous expression of P.68 or a modified version thereof according to the invention, or of P.94 or a modified version thereof according to the

invention, may be achieved. These proteins may be expressed therefore in a host which is not B.bronchiseptica. The proteins can thus be obtained free of any other components of B.bronchiseptica. The P.94 or modified version thereof may be processed to P.68 or a modified version thereof. P.68 or a modified version thereof can therefore be obtained from a host cell transformed with an expression vector containing a DNA sequence encoding P.68 or a modified version thereof or a DNA sequence encoding the precursor molecule P.94 or a modified version thereof.

The protein obtained in this way may be insoluble and thus may need to be refolded following the use of guanidinium hydrochloride as denaturant in conventional manner and in any event is preferably purified.

The invention additionally provides a veterinary composition comprising a protein of the invention and a veterinarily acceptable carrier or diluent. This composition may be used as a vaccine. The vaccine of the invention may optionally contain additional antigens of B.bronchiseptica.

The vaccine of the invention is normally associated with a veterinarily acceptable vehicle which allows the protein to be administered to an animal. Administration is usually carried out via the oral, intranasal, or preferably parenteral route. In the case of the parenteral route, the vehicle is generally liquid and the antigen is generally dissolved or suspended in it. An example of a liquid vehicle is physiological saline solution.

The vaccine may also contain an adjuvant for stimulating the immune response and thereby enhancing the potency of the antigen. Convenient adjuvants for use in the present invention include, for example aluminium hydroxide and aluminium phosphate.

Conveniently the vaccine contains a final concentration of protein in the range of from 0.01 to

5mg/ml, preferably 0.03 to 2mg/ml, most preferably 0.3mg/ml. After formulation the vaccine may be incorporated into a sterile container which is then sealed and stored at a low temperature, for example 4°C, or is freeze dried.

The invention also provides a method for inducing immunity to B.bronchiseptica in animals, comprising the administration to an animal of an effective amount of a protein of the present invention. The antigen of the invention may therefore be used to induce immunity to B.bronchiseptica - induced atrophic rhinitis in pigs. In order to induce immunity one or more doses of the vaccine are normally administered. Each dose of the vaccine may be from 1ml to 5ml, preferably 2 to 4ml.

The following Examples illustrate the invention. In the accompanying Figures:

Figure 1 shows the DNA sequence of the prn gene encoding the P.68 pertactin from B.bronchiseptica. Downward arrows indicate the sites of two possible protein cleavage regions that result in the production of the mature polypeptide. Arrows over protein sequence denote two protein repeat motifs: (i) $(GGXXP)_3$ encoded by nucleotides 939-983 and (ii) $(PQP)_7$ encoded by nucleotides 1852 and 1947. Two RGD tripeptide sequences encoded by nucleotides 922-930 and 2245-2253 are shown overlined although only the first sequence occurs in the mature protein. An inverted repeat that is followed by a run of T-residues is shown between residues 2898-2909 and resembles a rho-independent transcription terminator.

Figure 2 is a line drawing representing the strategy used to generate the P.68 expression plasmid pBD881. Plasmids pBD875 and pBD856 contain the 5′ and 3′ sections of the gene encoding B.bronchiseptica prn respectively and were used to generate the full-length gene encoding P.94. This full-length (non-expressing) gene harboured by pBD876 was excised by digestion with AflII and HindIII and ligated

with Nco1-HindIII digested pKK233-2 so as to generate the P.68 expressing plasmid pBD881.

EXAMPLE

1. Methods

Bacterial strains, plasmids and phage. B. bronchiseptica strain CN7531, was from the Wellcome culture collection (Wellcome Biotech, UK). E. coli K12 strains TG1 and HB101 were as previously described (Carter et al., 1985; Boyer & Roulland Dussoix, 1969). Cosmid pHC79 (Hohn & Collins, 1980) was from Amersham International (Little Chalfont, Buckinghamshire, UK). Plasmid pKK233-2 (Amann & Brosius, 1985) and M13 mp18 and M13 mp19 (Yanisch-Perron et al., 1985) were supplied by Pharmacia (Milton Keynes, Buckinghamshire, UK). E. coli strains were grown in Luria broth (LB) or LB solidified with 1-6% (w/v) agar (Millar, 1972). B. bronchiseptica was grown in Stainer-Scholte (Stainer & Scholte, 1962) broth or medium as previously described (Novotny et al., 1985a).

DNA isolation and manipulations. B. bronchiseptica CN7531 chromosomal DNA was prepared as described previously (Hull et al., 1981). Plasmid and bacteriophage DNA were isolated and purified by standard methods (Maniatis et al., 1982). All DNA-modifying enzymes were from Gibco BRL (Paisley, Scotland).

Construction of a B. bronchiseptica genomic library. Cosmid pHC79 DNA was digested with BamHI and ligated with Sau3A digested B. bronchiseptica DNA in the 40-50kb size range as described previously (Charles et al., 1990). The gene bank was plated out and 480 randomly selected colonies transferred to microtitre plates as described previously (Charles et al., 1990). Colonies were transferred to Gene Screen plus hybridization membranes (Du Pont, Stevenage, Hertford, UK), and hybridized with a ^{32}P-labelled ClaI fragment of the prn gene encoding the P.70 protein from B.parapertussis (International Application No.

PCT/GB91/02302) as described previously.

Subcloning and DNA sequencing. Recombinant phage DNA was sequenced after ligation of specific restriction endonuclease fragments of cosmid into the vector M13 mp18 and M13 mp19. Sequencing was carried out using universal primer [^{35}S] dATP and both gradient and wedge gels (Biggin et al., 1983; Sanger et al., 1977). Some clones were sequenced with modified T7 DNA polymerase (Tabor & Richardson, 1987) and 7-deaza-2-dGTP (Mizusawa et al., 1986) using a kit supplied by Pharmacia. Gaps in the sequence were filled in using synthetic oligonucleotides made on a MilliGen 7500 DNA synthesizer (Millipore, UK) as specific primers (Charles et al., 1986).

Immunological characterization of recombinant P.68. E. coli lysates harbouring recombinant P.68 protein were subjected to SDS-PAGE electrophoresis (Maniatis et al., 1982) and were transferred to nitrocellulose (Towbin et al., 1979). The monoclonal antibody BB05 (Montaraz et al., 1985; Novotny et al., 1985a) was used to detect P.68 protein using horse-radish peroxidase conjugated goat anti-mouse second antibody and 4-chloro-1-naphthol as substrate (Fairweather et al., 1986). To test for surface expression of recombinant P.68, slide agglutination assays were carried out as described in International Application No. PCT/GB91/02302. More specifically, samples of E. coli strains to be tested (10^6-10^7) were mixed with 30µl of IgG purified polyclonal rabbit anti-P.69 antibody on a glass microscope slide and visually scored for clumping after 2 mins as compared with a negative control of either vir$^-$ B. parapertussis or E. coli TG1.

2. Results

Isolation of the gene encoding the P.68 antigen. The B.bronchiseptica genomic library was screened by hybridisation with a radioactively labelled 1.8kb ClaI fragment of the prn gene encoding the P.70 antigen from

B.parapertussis. Of the two cosmids returning a positive signal one, designated pBD844, was selected for further analysis. Restriction mapping and Southern blotting experiments carried out on pBD844 demonstrated that the ClaI and SalI hybridization pattern of the prn gene from B.bronchiseptica was identical to that described for both B.pertussis (Charles et al., 1989) and B.parapertussis (International Application No. PCT/GB91/02302). These ClaI and SalI fragments were therefore assumed to contain the entire prn gene encoding the P.68 protein and were cloned into Bluescript pBSSKII$^+$ to generate pBD875 (ClaI) and pBD856 (SalI); they were also cloned into M13 mp18 and M13 mp19 for DNA sequencing.

Nucleotide sequence of the prn gene encoding P.68 DNA sequence was obtained initially from the ClaI and SalI fragments cloned into M13 and using universal primer; overlapping sequence was completed using a set of synthetic oligonucleotides as specific sequence primers. Computer analysis of the DNA sequence (Fig. 1) demonstrates that the open reading frame for the prn gene of P.68 protein is capable of encoding a protein with a Mr of 93,996 (P.94). The molecular weight of the mature protein found on the surface of B.bronchiseptica is however 68,000 as judged by SDS-PAGE, suggesting that this molecule is the processed form of the larger precursor. This processing is likely to involve the removal of both N-terminal and C-terminal sequences. N-terminal protein sequencing of purified preparations of P.68 from B.bronchiseptica gave the sequence Asp-Trp/Gln-Asn-Asn-Gln-Gln/Ser-Ile-Xaa-Lys-Ala confirming that a signal peptide is cleaved. Cleavage of this signal peptide, of between 32 or 34 amino acid residues depending on which ATG initiation codon is used, occurs after the sequence AYA which is in agreement with the Ala-Xaa-Ala motif reported to be recognized by E. coli signal peptidases (Perlman & Halvorson, 1983). Towards the C-terminus of the P.68 protein at residues 600-602 is a

dibasic pair of amino acids, Lys-Arg, that occur in the same position, and are flanked by identical residues.

Examination of the first 140bp of the DNA sequence in Fig. 1 shows that there are no good fits with either the consensus ribosome binding site (Shine & Dalgarno, 1975) or consensus promoter elements identified for E. coli genes (Rosenberg & Court, 1979). There is an inverted repeat after the stop codon, that is followed by a run of T-residues.

Heterologous expression of P.68 in E. coli. Cosmid pBD844 harbouring the B.bronchiseptica prn gene was unable to direct the expression of P.68 in E. coli. In order to express the gene in E. coli the entire structural gene was cloned into the expression plasmid pKK233-2. Fig. 2 outlines the expression strategy; an EcoR1-EcoRV fragment comprising the 5' end of P.68 prn from plasmid pBD875 was ligated into EcoRV-EcoRI digested pBD856 (containing the 3' end of P.68 prn) so as to generate pBD876 containing the entire structural gene. Plasmid pBD876 was digested with AflIII-HindIII and ligated with NcoI-HindIII digested pKK233-2. This ligation mixture was used to transform E. coli TG1 and transformants expressing P.68 were identified by their ability to cross-react with the mAb BB05 in protein dot blots.

Colonies returning a positive signal were isolated and plasmid minipreps carried out to verify that a full-length insert has been cloned. One such plasmid, pBD881, was selected for further study. A Western blot of an SDS-PAGE gel of E. coli TG1 harbouring pBD881 surprisingly did not produce a detectable 94kDa higher molecular weight band, but instead produces a pair of stronger bands at around 69kDa and 68kDa.

To test whether or not the P.68 protein was expressed on the surface of E. coli, slide agglutination assays were carried out using both monoclonal antibody BB05 and polyclonal anti-P.69 antibody. Agglutination occurred just

as rapidly with E. coli harbouring pDB881 as with vir+ B.bronchiseptica, suggesting that the heterologous protein is surface located.

REFERENCE EXAMPLE

Cloning of B. parapertussis chromosomal DNA into cosmid pHC79 and identification of the prn gene encoding P.70

B. parapertussis chromosomal DNA (prepared by the method of Hull et al, 1981 as modified by Maskell et al, 1988) was partially digested with Sau3A, and fragments in the 40-50kb size range were ligated into the BamHI site (Maniatis et al, 1982) of cosmid pHC79 (Hohn & Collins, 1980). Recombinant cosmids in E. coli HB101 were plated out and transferred to microtiter plates following the method described by Charles et al, 1990 and transferred to Gene Screen Plus hybridized membranes (Du Pont, Stevenage, Hertfordshire). The cosmids were then screened for the presence of the prn gene encoding P.70 by DNA : DNA hybridization using a radioactively labelled 1.8kb ClaI restriction fragment isolated from the related prn gene from B. pertussis. The ClaI fragment was gel purified (Tautz & Renz, 1983) following digestion of cosmid pI69 (Charles et al, 1989). The fragment was nick translated with a kit supplied by BCL, Mannheim and [α^{32}P]-ATP (Amersham), and hybridized with the B. parapertussis gene bank filters as previously described (Charles et al, (1990)). Three positive colonies were identified and one, harbouring cosmid pBD811 was selected for further analysis.

Subcloning and DNA sequencing

Restriction fragments of cosmid pBD811 were cloned in M13mp18 and M13mp19 (Yanisch-Perron et al, 1985) and sequenced using universal primer, [α^{-35}S] dATP (deoxyadenosine 5'-[α^{35}S] thiotriphosphate) dideoxynucleotide triphosphates, and both gradient and wedge gels (Biggin et al, 1983; Sanger et al, 1977). To

resolve compression artifacts some clones were sequenced with modified T7 DNA polymerase (Tabor & Richardson, 1987) and 7-deaza-2'-dGTP (Mizusawa et al, 1986) using a kit supplied by Pharmacia. Gaps in the sequence were filled in using synthetic oligonucleotides as specific primers (Charles et al, 1986).

Oligonucleotides for use as specific sequencing primers were made on a SAM1 oligonucleotide synthesizer (Biolabs).

Computer analysis of the DNA sequence revealed an open reading frame capable of encoding a protein of 922 amino acids with a calculated molecular weight of 95,177.

References

AMMAN, E. & BROSIUS, J. (1985). 'ATG' vectors for regulated high-level expression of cloned genes in Escherichia coli. Gene 40, 183-190.

BIGGIN, M. D., GIBSON, T. J. & HONG, G. F. (1983). Buffer gradient gels and ^{35}S label as an aid to rapid DNA sequence determination. Proceedings of the National Academy of Sciences of the United States of America 80, 3963-3965.

BOYER, H. W. & ROULLAND-DUSSOIX, D. (1969). A complementation analysis of the restriction and modification of DNA in Escherichia coli. Journal of Molecular Biology 41, 459-465.

CARTER, P., BEDOUELL, H. & WINTER, G. (1985). Improved oligonucleotide site directed mutagenesis using M13 vectors. Nucleic Acids Research 13, 443-444.

CHARLES, I. G., KEYTE, J., BRAMMAR, W. J., SMITH, M. & HAWKINS, A. R. (1986). The isolation and nucleotide sequence of the complex AROM locus of Aspergillus nidulans. Nucleic Acids Research 14, 2201-2213.

CHARLES, I. G., DOUGAN, G., PICKARD, D., CHATFIELD, S., SMITH M., NOVOTNY, P., MORRISSEY, P. & FAIRWEATHER, N. F. (1989). Molecular cloning and characterization of protective outer membrane protein P.69 from Bordetella pertussis. Proceedings of the National Academy of Sciences of the United States of America 86, 3554-3558.

CHARLES, I. G., LAMB, H. K., PICKARD, D., DOUGAN, G. & HAWKINS, A. R. (1990). Isolation, characterization and nucleotide sequences of the aroC genes encoding chorismate synthase from Salmonella typhi and Escherichia coli. Journal of General Microbiology 136, 353-358.

FAIRWEATHER, N. F., LYNESS, V. A., PICKARD, D., ALLEN, G. & THOMSON. R. O. (1986). Cloning, nucleotide sequencing and expression of tetanus toxin fragment C in Escherichia coli. Journal of Bacteriology 165, 21-27.

HOHN, B. & COLLINS, J. (1980). A small cosmid for efficient cloning of large DNA fragments. Gene 11, 291-

298.

HULL, R. A., GILL, R. E., HSU, P., MINSHEW, B. H. & FALKOW, S. (1981). Construction and expression of recombinant plasmids encoding type 1 of D-mannose resistant pili from a urinary tract infection Escherichia coli. Infection and Immunity 33, 933-938.

KOBISCH, M. & NOVOTNY, P. (1990). Identification of a 68-kilodalton outer membrane protein as the major protective antigen of Bordetella bronchiseptica by using specific-pathogen-free piglets. Infection and Immunity 58, 352-357.

MANIATIS, T., FRITSCH, E. E. & SAMBROOK, J. (1982). Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory. Cold Spring Harbor, N.Y.

MASKELL et al, J. Bacteriol. 170, 2467-2471, 1988

MILLER, J. M. (1972). Experiments in Molecular Genetics. Cold Spring Harbor N.Y.: Cold Spring Harbor Laboratory.

MONTARAZ, J. A., NOVOTNY, P. & IVANYI, J. (1985) Identification of a 68-kilodalton protective antigen from Bordetella bronchiseptica. Infection and Immunity 47, 744-751.

MIZUSAWA, S., NISHIMURA, S. & SEELA, F. (1986). Improvements of the dideoxy chain termination method of DNA sequencing by use of deoxy-7-deazaguanosine triphosphate in place of dGTP. Nucleic Acids Research 14, 1319-1324.

NOVOTNY, P., CHUBB, A. P., COWNLEY, K., MONTARAZ, J. A & BEESLEY, J. E. (1985a). Bordetella adenylate cyclase: A genus specific protective antigen and virulence factor. In Proceedings of the Fourth International Symposium on Pertussis. Development of Biological Standards 61, 27-41.

NOVOTNY, P., KOBISCH, M., COWNLEY, K., CHUBB, A. P. & MONTARAZ, J. A. (1985b). Evaluation of Bordetella bronchiseptica vaccines in specific-pathogen-free piglets with bacterial cell surface antigens in enzyme-linked immunosorbent assay. Infection and Immunity 50, 190-198.

PERLMAN, D. & HALVORSON, H. O. (1983). A putative signal peptidase recognition site and sequence in eukaryotic and

prokaryotic peptides. Journal of Molecular Biology 167, 391-409.

ROSENBERG, M. & COURT, D. (1979). Regulation sequences involved in the promotion and termination of RNA transcription. Annual Review of Genetics 13, 319-353.

SANGER, F., NICKLEN, S. & COULSON, A. R. (1977). DNA sequencing with chain terminating inhibitors. Proceedings of the National Academy of Sciences of the United States of America 71, 5463-5467.

SHINE, J. & DALGARNO, 1. (1975). Determinant of cistron specificity in bacterial ribosomes. Nature 254, 34-38.

STAINER, D.W. & SCHOLTE, M. J. (1962). A simple chemically defined medium for the production of phase I Bordetella pertussis. Journal of General Microbiology 63, 211-220.

TABOR, S. & RICHARDSON, C. C. (1987). DNA sequence analysis with a modified bacteriophage T7 polymerase. Proceedings of the National Academy of Sciences in the United States of America 84, 4767-4771.

TAUTZ & RENZ, Anal. Biochem. 132, 14-19, 1983

TOWBIN J., STAEHLIN, R. & GORDON, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedures and some applications. Proceedings of the National Academy of Sciences of the United States of America 76, 4350-4354.

YANISCH-PERRON, C., VIEIRA, J. & MESSING. J. (1985). Improved M13 phage vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33, 103-119.

CLAIMS

1. A protein which is uncontaminated by components from B. bronchiseptica, which is capable of binding to antibody which also binds the native P.68 antigen of B. bronchiseptica and which has (a) the amino acid sequence:

DWNNQSIIKAGERQHGIHIKQSDGAGVRTATGTTIKVSGRQAQGVLLENPAAELRFQNG
SVTSSGQLFDEGVRRFLGTVTVKAGKLVADHATLANVSDTRDDDGIALYVAGEQAQASI
ADSTLQGAGGVRVERGANVTVQRSTIVDGGLHIGTLQPLQPEDLPPSRVVLGDTSVTAV
PASGAPAAVSVFGANELTVDGGHITGGRAAGVAAMDGAIVHLQRATIRRGDAPAGGAVP
GGAVPGGFGPLLDGWYGVDVSDSTVDLAQSIVEAPQLGAAIRAGRGARVTVSGGSLSAP
HGNVIETGGGARRFPPPASPLSITLQAGARAQGRALLYRVLPEPVKLTLAGGAQGQGDI
VATELPPIPGASSGPLDVALASQARWTGATRAVDSLSIDNATWVMTDNSNVGALRLASD
GSVDFQQPAEAGRFKCLMVDTLAGSGLFRMNVFADLGLSDKLVVMRDASGQHRLLVRNS
GSEPASGNTMLLVQTPRGSAATFTLANKDGKVDIGTYRYRLAANGNGQWSLVGAKAPPA
PKPAPQPGPQPGPQPPQPPQPPQPPQRQPEAPAPQPPAGRELSAAANAAVNTGGVGLAS
TLWYAESN

or (b) an amino acid sequence which has a homology of more than 98% with the said amino acid sequence (a).

2. A DNA sequence which encodes a protein as defines in claim 1.

3. A DNA sequence according to claim 2, which consists essentially of the nucleotide sequence shown in Figure 1 from nucleotide 247 to nucleotide 2040.

4. A DNA sequence according to claim 2, which differs from the nucleotide sequence shown in Figure 1 from nucleotide 247 to nucleotide 2040 at no more than twelve positions.

5. A protein which as the amino acid sequence shown in Figure 1 or an amino acid sequence which has a homology of more than 98% with the amino acid sequence shown in Figure 1.

6. A DNA sequence which encodes a protein as defined in claim 5.

7. A DNA sequence according to claim 6, which consists essentially of the nucleotide sequence shown in Figure 1 from nucleotide 145 to nucleotide 2877.

8. A DNA sequence according to claim 6, which differs from the nucleotide sequence shown in Figure 1 from nucleotide 145 to 2877 at no more than twelve positions.

9. A DNA sequence which consists essentially of the sequence shown in Figure 1 or a sequence which has a homology of more than 98% with the sequence shown in Figure 1.

10. An expression vector which contains a DNA sequence as defined in any one of claims 2 to 4 and 6 to 9, and which, when provided in a suitable host, is capable of expressing a protein as defined in claim 1 or 5.

11. A vector according to claim 10, which is a plasmid.

12. A host transformed with an expression vector as defined in claim 10 or 11.

13. A host according to claim 12, which is a strain of E. coli.

14. A process for the preparation of a protein as defined in claim 1, which process comprises maintaining a host as claimed in claim 12 or 13 under such conditions that said protein is expressed.

15. A process for the preparation of a protein as defined in claim 5, which process comprises maintaining a host as claimed in claim 12 or 13, which has been transformed by an expression vector as claimed in claim 10 which contains a DNA sequence as defined in any one of claims 6 to 9, under such conditions that the said protein is expressed.

16. A veterinary composition comprising a veterinarily acceptable carrier or diluent and, as active ingredient, a protein as defined in claim 1 or 5.

Fig.1 (1/3)

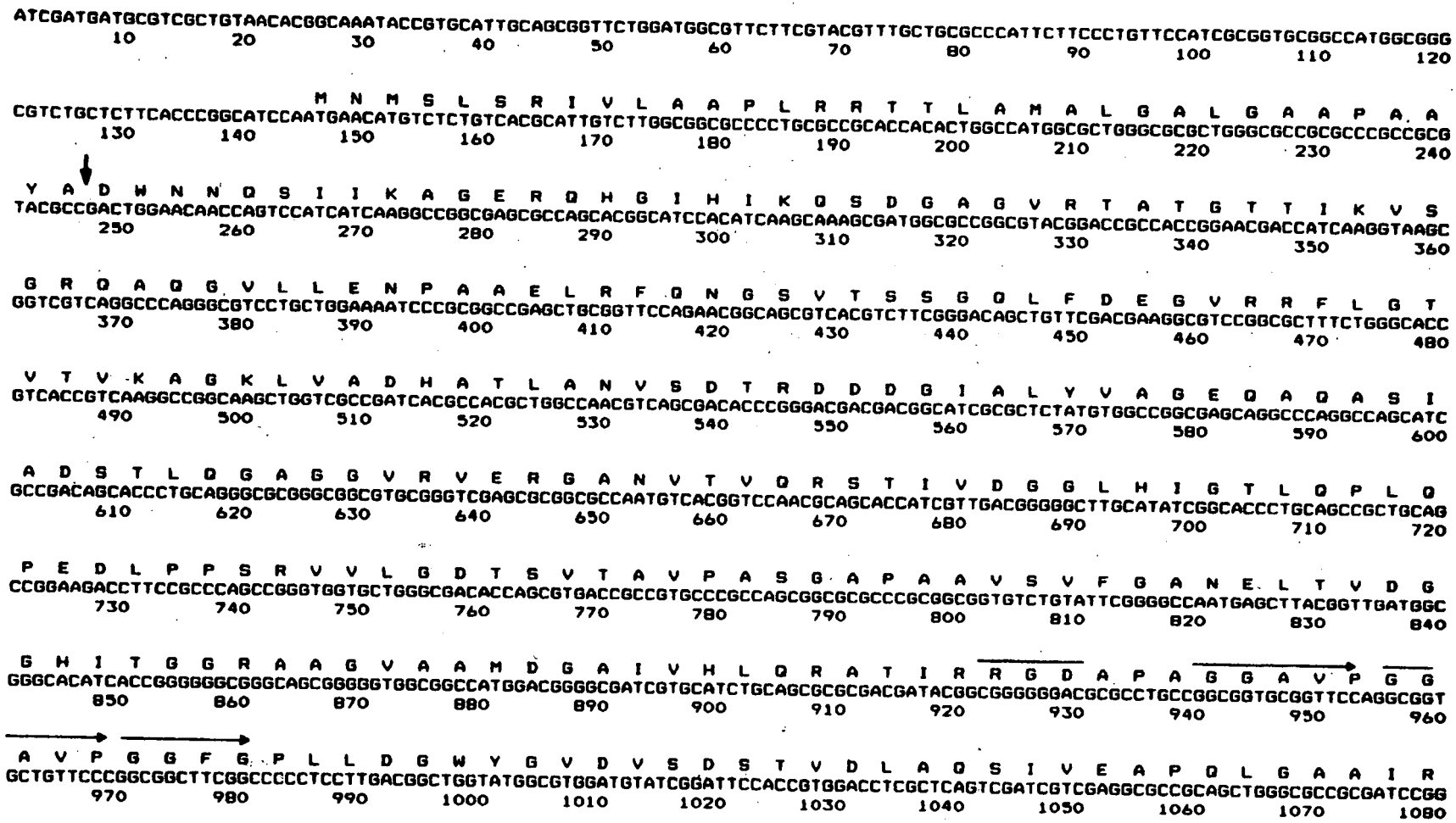

ATCGATGATGCGTCGCTGTAACACGGCAAATACCGTGCATTGCAGCGGTTCTGGATGGCGTTCTTCGTACGTTTGCTGCGCCCATTCTTCCCTGTTCCATCGCGGTGCGGCCATGGCGGG
10 20 30 40 50 60 70 80 90 100 110 120
M N M S L S R I V L A A P L R R T T L A M A L G A L G A A P A A
CGTCTGCTCTTCACCCGGCATCCAATGAACATGTCTCTGTCACGCATTGTCTTGGCGGCGCCCCTGCGCCGCACCACACTGGCCATGGCGCTGGGCGCGCTGGGCGCCGCGCCCGCCGCG
130 140 150 160 170 180 190 200 210 220 230 240
Y A D W N N Q S I I K A G E R Q H G I H I K Q S D G A G V R T A T G T T I K V S
TACGCCGACTGGAACAACCAGTCCATCATCAAGGCCGGCGAGCGCCAGCACGGCATCCACATCAAGCAAAGCGATGGCGCCGGCGTACGGACCGCCACCGGAACGACCATCAAGGTAAGC
250 260 270 280 290 300 310 320 330 340 350 360
G R Q A Q G V L L E N P A A E L R F Q N G S V T S S G Q L F D E G V R R F L G T
GGTCGTCAGGCCCAGGGCGTCCTGCTGGAAAATCCCGCGGCCGAGCTGCGGTTCCAGAACGGCAGCGTCACGTCTTCGGGACAGCTGTTCGACGAAGGCGTCCGGCGCTTTCTGGGCACC
370 380 390 400 410 420 430 440 450 460 470 480
V T V K A G K L V A D H A T L A N V S D T R D D D G I A L Y V A G E Q A Q A S I
GTCACCGTCAAGGCCGGCAAGCTGGTCGCCGATCACGCCACGCTGGCCAACGTCAGCGACACCCGGGACGACGACGGCATCGCGCTCTATGTGGCCGGCGAGCAGGCCCAGGCCAGCATC
490 500 510 520 530 540 550 560 570 580 590 600
A D S T L Q G A G G V R V E R G A N V T V Q R S T I V D G G L H I G T L Q P L Q
GCCGACAGCACCCTGCAGGGCGCGGGCGGCGTGCGGGTCGAGCGCGGCGCCAATGTCACGGTCCAACGCAGCACCATCGTTGACGGGGGCTTGCATATCGGCACCCTGCAGCCGCTGCAG
610 620 630 640 650 660 670 680 690 700 710 720
P E D L P P S R V V L G D T S V T A V P A S G A P A A V S V F G A N E L T V D G
CCGGAAGACCTTCCGCCCAGCCGGGTGGTGCTGGGCGACACCAGCGTGACCGCCGTGCCCGCCAGCGGCGCGCCCGCGGCGGTGTCTGTATTCGGGGCCAATGAGCTTACGGTTGATGGC
730 740 750 760 770 780 790 800 810 820 830 840
G H I T G G R A A G V A A M D G A I V H L Q R A T I R R G D A P A G G A V P G G
GGGCACATCACCGGGGGGCGGGCAGCGGGGGTGGCGGCCATGGACGGGGCGATCGTGCATCTGCAGCGCGCGACGATACGGCGGGGGGACGCGCCTGCCGGCGGTGCGGTTCCAGGCGGT
850 860 870 880 890 900 910 920 930 940 950 960
A V P G G F G P L L D G W Y G V D V S D S T V D L A Q S I V E A P Q L G A A I R
GCTGTTCCCGGCGGCTTCGGCCCCCTCCTTGACGGCTGGTATGGCGTGGATGTATCGGATTCCACCGTGGACCTCGCTCAGTCGATCGTCGAGGCGCCGCAGCTGGGCGCCGCGATCCGG
970 980 990 1000 1010 1020 1030 1040 1050 1060 1070 1080

Fig.1. (2/3)

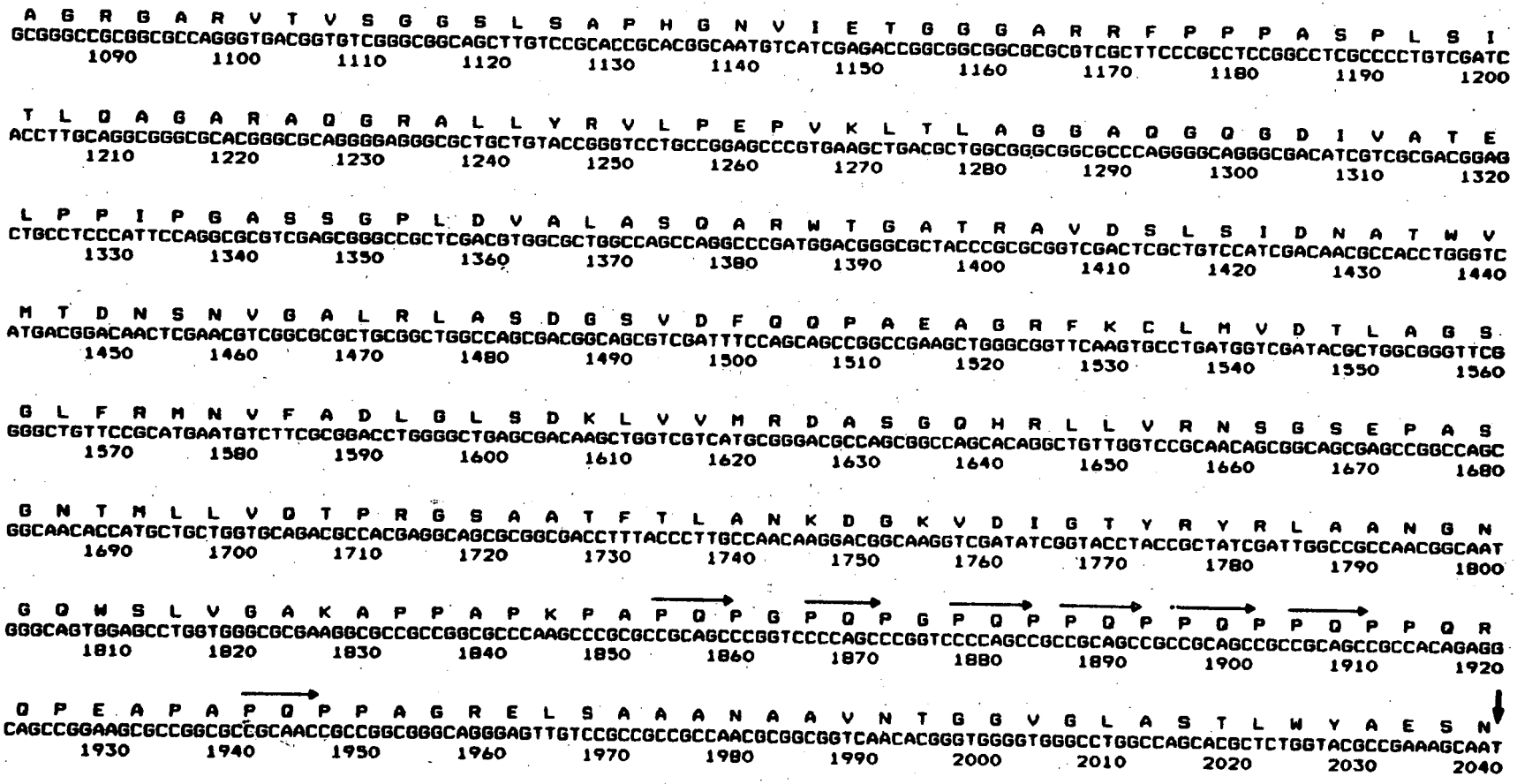

A G R G A R V T V S G G S L S A P H G N V I E T G G G A R R F P P P A S P L S I
GCGGGCCGCGGCGCCAGGGTGACGGTGTCGGGCGGCAGCTTGTCCGCACCGCACGGCAATGTCATCGAGACCGGCGGCGGCGCGCGTCGCTTCCCGCCTCCGGCCTCGCCCCTGTCGATC
1090 1100 1110 1120 1130 1140 1150 1160 1170 1180 1190 1200
T L Q A G A R A Q G R A L L Y R V L P E P V K L T L A G G A Q G Q G D I V A T E
ACCTTGCAGGCGGGCGCACGGGCGCAGGGGAGGGCGCTGCTGTACCGGGTCCTGCCGGAGCCCGTGAAGCTGACGCTGGCGGGCGGCGCCCAGGGGCAGGGCGACATCGTCGCGACGGAG
1210 1220 1230 1240 1250 1260 1270 1280 1290 1300 1310 1320
L P P I P G A S S G P L D V A L A S Q A R W T G A T R A V D S L S I D N A T W V
CTGCCTCCCATTCCAGGCGCGTCGAGCGGGCCGCTCGACGTGGCGCTGGCCAGCCAGGCCCGATGGACGGGCGCTACCCGCGCGGTCGACTCGCTGTCCATCGACAACGCCACCTGGGTC
1330 1340 1350 1360 1370 1380 1390 1400 1410 1420 1430 1440
M T D N S N V G A L R L A S D G S V D F Q Q P A E A G R F K C L M V D T L A G S
ATGACGGACAACTCGAACGTCGGCGCGCTGCGGCTGGCCAGCGACGGCAGCGTCGATTTCCAGCAGCCGGCCGAAGCTGGGCGGTTCAAGTGCCTGATGGTCGATACGCTGGCGGGTTCG
1450 1460 1470 1480 1490 1500 1510 1520 1530 1540 1550 1560
G L F R M N V F A D L G L S D K L V V M R D A S G Q H R L L V R N S G S E P A S
GGGCTGTTCCGCATGAATGTCTTCGCGGACCTGGGGCTGAGCGACAAGCTGGTCGTCATGCGGGACGCCAGCGGCCAGCACAGGCTGTTGGTCCGCAACAGCGGCAGCGAGCCGGCCAGC
1570 1580 1590 1600 1610 1620 1630 1640 1650 1660 1670 1680
G N T M L L V Q T P R G S A A T F T L A N K D G K V D I G T Y R Y R L A A N G N
GGCAACACCATGCTGCTGGTGCAGACGCCACGAGGCAGCGCGGCGACCTTTACCCTTGCCAACAAGGACGGCAAGGTCGATATCGGTACCTACCGCTATCGATTGGCCGCCAACGGCAAT
1690 1700 1710 1720 1730 1740 1750 1760 1770 1780 1790 1800
G Q W S L V G A K A P P A P K P A P Q P G P Q P G P Q P P Q P P Q P P Q P P Q R
GGGCAGTGGAGCCTGGTGGGCGCGAAGGCGCCGCCGGCGCCCAAGCCCGCGCCGCAGCCCGGTCCCCAGCCCGGTCCCCAGCCGCCGCAGCCGCCGCAGCCGCCGCAGCCGCCACAGAGG
1810 1820 1830 1840 1850 1860 1870 1880 1890 1900 1910 1920
Q P E A P A P Q P P A G R E L S A A A N A A V N T G G V G L A S T L W Y A E S N
CAGCCGGAAGCGCCGGCGCCGCAACCGCCGGCGGGCAGGGAGTTGTCCGCCGCCGCCAACGCGGCGGTCAACACGGGTGGGGTGGGCCTGGCCAGCACGCTCTGGTACGCCGAAAGCAAT
1930 1940 1950 1960 1970 1980 1990 2000 2010 2020 2030 2040

Fig.1. (3/3)

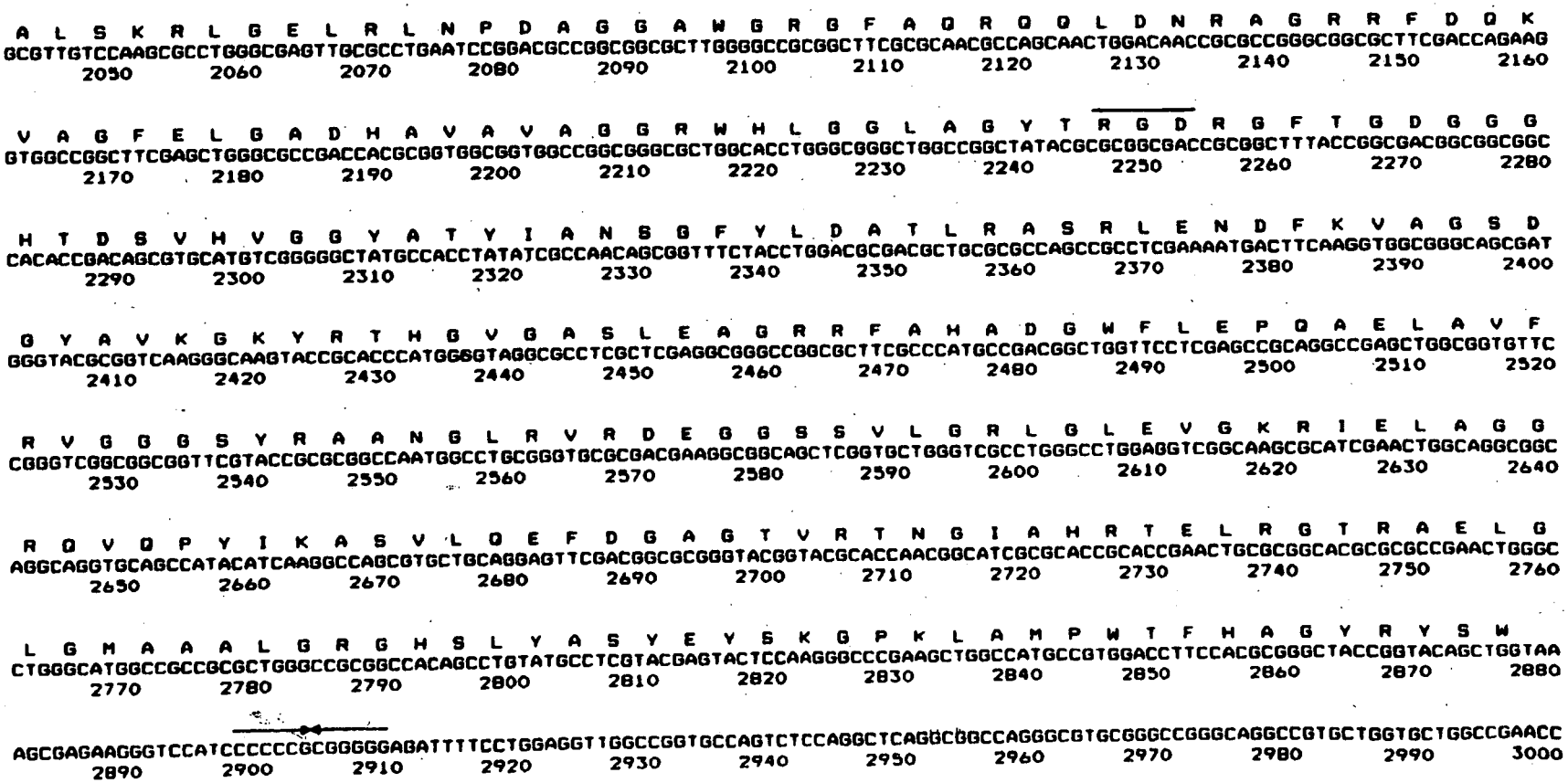


Fig.2.

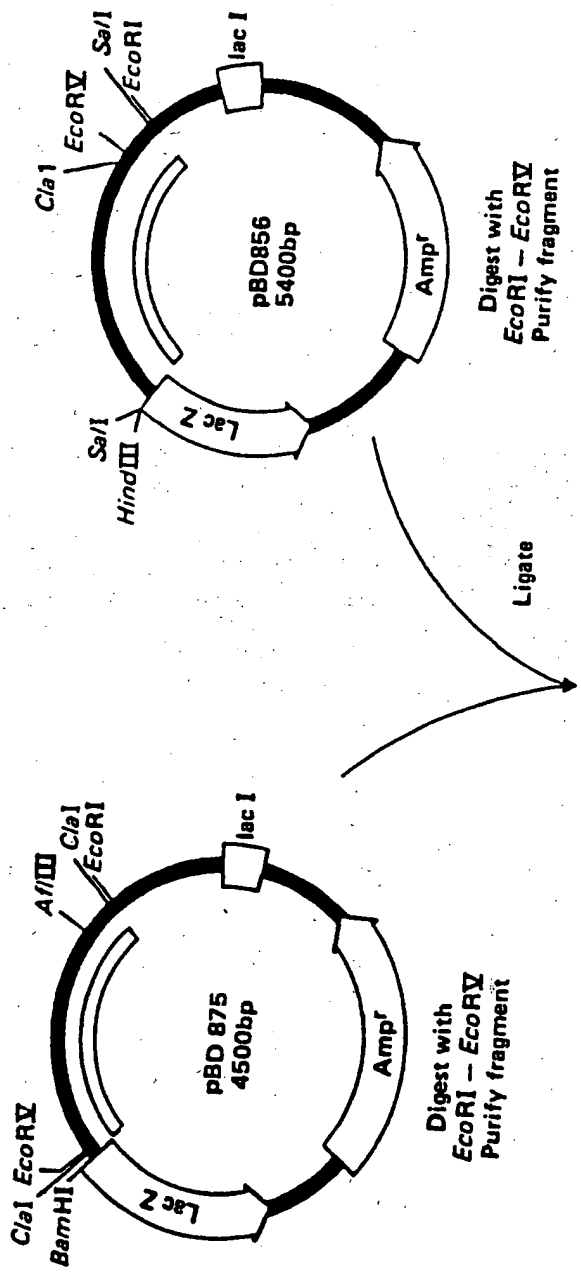
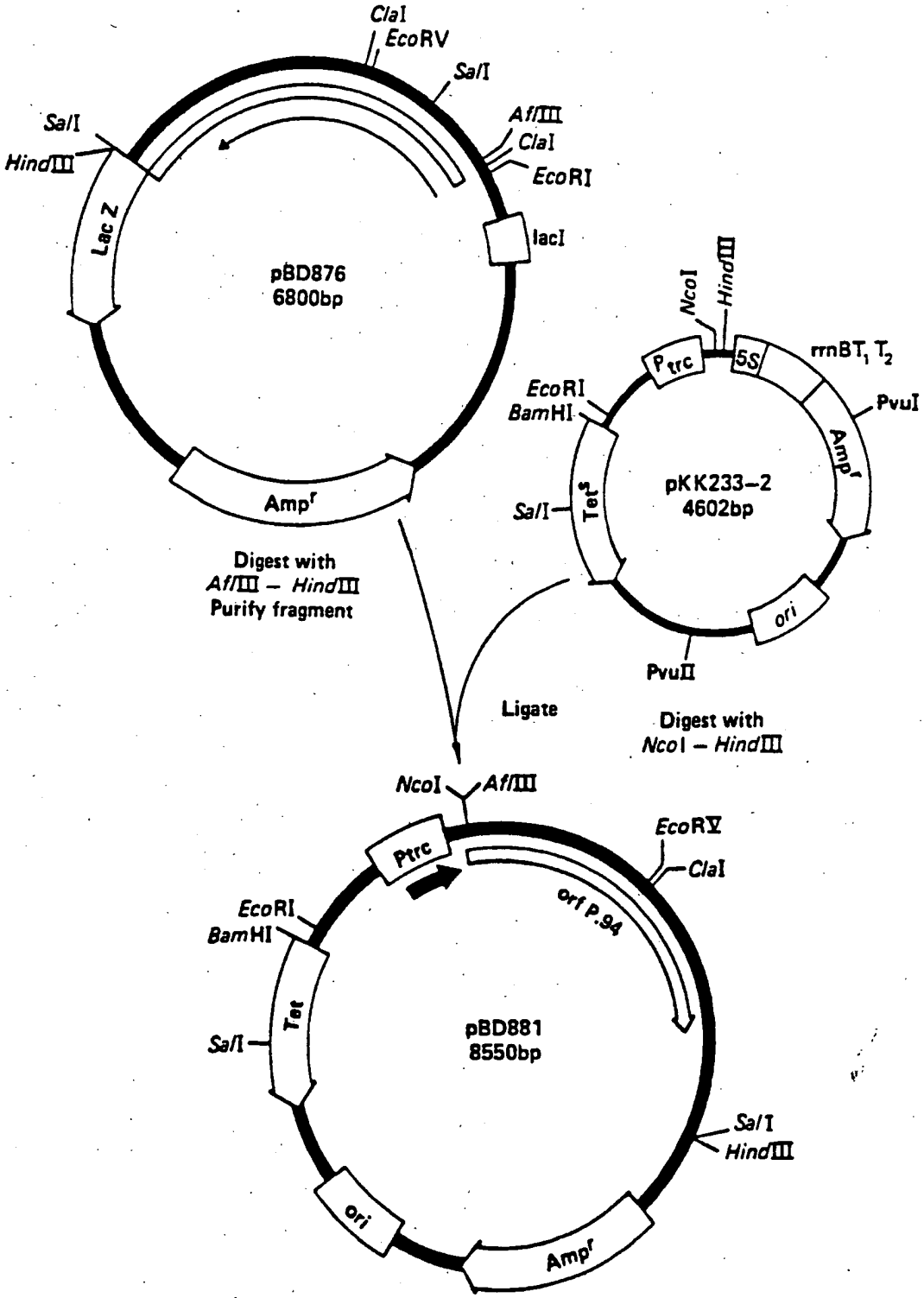

Sal I
EcoRI
lac I
EcoRV
Cla I
pBD856
5400bp
Ampr
Digest with
EcoRI – EcoRV
Purify fragment
Lac Z
Sal I
HindIII
Ligate
Af/III
Cla I
EcoRI
lac I
pBD 875
4500bp
Ampr
Digest with
EcoRI – EcoRV
Purify fragment
Cla I
EcoRV
BamHI
Lac Z

Fig. 2. cont.


ClaI
EcoRV
SalI
AflIII
ClaI
EcoRI
SalI
HindIII
Lac Z
lacI
pBD876
6800bp
Ampr
Digest with
AflIII – HindIII
Purify fragment
NcoI
HindIII
Ptrc
5S
rrnBT1 T2
EcoRI
BamHI
PvuI
SalI
Tets
Ampr
pKK233–2
4602bp
ori
PvuII
Digest with
NcoI – HindIII
Ligate
NcoI
AflIII
EcoRV
Ptrc
ClaI
EcoRI
BamHI
orf P.94
Tet
SalI
pBD881
8550bp
SalI
HindIII
ori
Ampr

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 92/00561

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/31; C07K13/00; C12N1/21; A61K39/10

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C07K ; C12N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
X	INFECTION AND IMMUNITY. vol. 47, no. 3, March 1985, WASHINGTON US pages 744 - 751; J. MONTAREZ ET AL.: 'Identification of a 68-kilodalton protective protein antigen from Bordetella bronchiseptica' see the whole document	1-16
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 86, 1989, WASHINGTON US pages 3554 - 3558; I. CHARLES ET AL.: 'Molecular cloning and characterization of protective outer membrane protein P.69 from Bordetella pertussis' see the whole document	1-16

-/--

° Special categories of cited documents : [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
10 JUNE 1992	17 JUN 1992
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	VAN DER SCHAAL C.A.

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	MOLECULAR MICROBIOLOGY vol. 5, no. 2, 22 February 1991, pages 409 - 418; L. LI ET AL.: 'P.70 pertactin, an outer-membrane protein from Bordetella parapertussis: Cloning, nucleotide sequence and surface expression in E. coli' see the whole document	1-16
P,X	WO,A,9 115 571 (THE WELLCOME FOUNDATION LTD) 17 October 1991 see the whole document	1-16

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. GB 9200561 SA 57816

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 10/06/92
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9115571	17-10-91	None	